

Remarks

Reconsideration of this Application is respectfully requested.

Applicants respectfully submit that entry of the foregoing amendment is proper, as it obviates the rejections set forth in the office action. Upon entry of the foregoing amendment, claims 75-79, 81-84, 86-103, 104-107, and 109-136 are pending in the application, with claims 75, 103, and 125 being the independent claims. Claims 80, 85, 104 and 108 are sought to be cancelled without prejudice to or disclaimer of the subject matter therein. New claims 126-136 are sought to be added. These changes are believed to introduce no new matter, and their entry is respectfully requested.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding objections and rejections and that they be withdrawn.

Description of the Invention

The invention provides novel expression vectors that permit tight regulation of gene expression in eukaryotic cells. More specifically, the invention provides DNA vectors comprising nucleotide sequences that are transcribed to form RNA molecules which are then replicated by a temperature-sensitive replicase to form additional RNA molecules. The RNA molecules produced by replication contain a nucleotide sequence which may be translated to produce a protein of interest or which encode one or more untranslated RNA molecules.

Rejections under 35 U.S.C. § 112

Claims 75-84, 86-101, 103-107, and 109-125 were rejected for alleged lack of an adequate written description. This rejection is obviated by the foregoing amendment and by the remarks set forth below.

The rejection is premised on the belief that the specification contains "subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention." Office Action, pg. 2. More particularly, the Office Action states that "the mutations disclosed cannot be considered to be applicable to any polymerase other than Sindbis virus polymerase." Office Action, pg. 4-5. Applicants note that, as amended, the claims specify that the polymerase is of alphaviral origin. Applicants respectfully submit that Applicants clearly had possession of the claimed invention as of the filing date.

As the Office Action acknowledges, the specification discloses the P726S nsP2 and G153E nsP4 mutations, which are structural features that render Sindbis virus polymerase temperature sensitive and non-cytopathic. Applicant respectfully submits that Applicant was in possession not only of the claimed nucleic acids of Sindbis viral origin, but also of nucleic acids of Alphaviral origin in general. Indeed, Alphaviruses generally share a very high degree of sequence homology. For example, Sindbis virus and Semliki Forest virus (SFV) share 60% sequence identity, and their nsp4 regions share 73% sequence identity. Mutation of the same glycine residue (Gly 153 in SFV nsp4, and Gly 154 in Sindbis nsp4) produces a temperature-sensitive phenotype. Additional temperature-sensitive mutations in the SFV RNA dependent RNA

polymerase also were known as of the filing date of the present application (see, e.g., Hahn *et al.*, *J. Virol.* 63:1194-1202 (1989); copy enclosed).

Other Alphaviruses, such as Aura virus, also share a high degree of sequence identity with Sindbis virus and can be used in the invention. For example, the nsp4 regions of Sindbis and Aura viruses share 80% sequence identity, and the nsp2 regions share 68% sequence identity. Likewise, the nsp4 regions of Sindbis virus and Venezuelan equine encephalitis virus share 72% sequence identity, and the nsp2 regions share 55% sequence identity. Additional non-cytopathic mutations were identified (see Perri *et al.*, *J. Virol.* 74:9802-9807 (2000), copy enclosed) using methods that were known as of the filing date (see Frolov *et al.*, *Proc. Natl. Acad. Sci. USA* 93:11371-11377 (1996), copy enclosed)). Thus, in view of the high degree of homology between Sindbis viruses and other Alphaviruses, the disclosed nucleic acid encoding a non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase is representative of the claimed genus, and Applicant had possession of the necessary common attributes of features of the elements possessed by members of the claimed genus in compliance with the written description requirement. Accordingly, this rejection should be withdrawn, which is respectfully requested.

Claims 75-84, 86-101, 103-107, and 109-125 were rejected for alleged lack of enablement. This rejection is obviated by the foregoing amendment and by the remarks set forth below.

The rejection is premised, in part, on the belief that the specification does not provide an enabling disclosure for the use of cells other than BHK21 cells. This basis for rejecting the claims should be withdrawn. As is established by the enclosed Declaration

of Dr. Marco Boorsma, the claimed nucleic acids encode an RNA-dependent RNA polymerase that is non-cytopathic and temperature sensitive in a wide variety of mammalian cell types, such as BHK cells, CHO-K1 cells, COS7 cells, HEK293 cells, HEK293T cells, BHK21 C13-2P cells, and C2C12 myoblasts. Additionally, the enclosed Declaration establishes that a pCYTts vector that lacks the ts6 temperature sensitive mutation is non-cytopathic and can be used to transfect cells, e.g. HEK293 cells, contrary to the allegations in Agapov *et al.*, *Proc. Natl. Acad. Sci. USA* 95:12989-12994 (1998). Accordingly, the claimed nucleic acids can be used in a variety of mammalian cells types without undue experimentation.

The rejection for lack of enablement was also based on the belief that "[t]he specification asserts no utility for using these nucleic acids *in vivo* other than gene therapy." Applicants respectfully disagree. The specification discloses, for example, that the "nucleic acid molecules of the invention [can] encode an untranslated RNA molecule, such as an antisense RNA molecule, tRNA molecules, rRNA molecule or ribozyme" (pg. 8, lines 11-13). Furthermore, "[w]hen these recombinant host cells are intended to express polypeptide or untranslated RNA sequences in an individual, the nucleic acid molecules of the present invention may be introduced into host cells either *in vivo* or *ex vivo*" (emphasis added; see pg. 9, lines 16-18; pg. 10, lines 3-8; pg. 29, lines 16-20; pg. 34, line 22; pg. 36, lines 6-22; and pg. 40, lines 10-14). "The invention further provides methods for regulating the expression of proteins or untranslated RNA molecules in an individual" (pg. 9, lines 24-26). Furthermore, a wide variety of nucleotide sequences of interest can be expressed, including prodrug converting enzymes, toxins, antigens which stimulate immune responses, single chain antibodies,

5/11/01 72

proteins which stimulate or inhibit immune responses, etc. Thus, the specification discloses numerous *in vivo* uses that would not be considered "gene therapy."

The Office Action also makes the generalized allegations that "gene therapy is highly unpredictable, known delivery and expression systems are inadequate for therapeutic purposes, and the instant specification fails to complement the deficiencies of the prior art." To the contrary, the specification discloses that inducible alphaviral vectors should be used to achieve gene expression, and the specification provides the necessary guidance with respect to how to achieve such gene expression. None of the generalized allegations set forth in the Office Actions specifically refute Applicants' evidence that the invention can be practiced as claimed by carrying out the methods disclosed in the specification.

Furthermore, in various embodiments, the invention features the *in vivo* use of cells that were transformed with the nucleic acids of the invention. The use of cells transformed with nucleic acids has been well documented. Moreover, advanced methods that were known as of the filing date, such as cellular encapsulation (see Sagot *et al.*, *Eur. J. Neurosci.* 7:131-1322 (1995)) represent a further application of the claimed nucleic acids which does not necessitate transfer of genetic material *in vivo*. Likewise, DNA vaccination methods (see Lindberg, *Curr. Opin. Microbiol.* 1:116-124 (1998)) represent an *in vivo* use of nucleic acids that is not "gene therapy." In sum, contrary to statement set forth in the Office Action, the *in vivo* use of the claimed nucleic acids molecules is not limited to gene therapy. Furthermore, a person of ordinary skill in the art would be able to make and use the claimed nucleic acids and methods without undue experimentation.

The rejection for alleged lack of enablement also was premised on the belief that "[o]ne of skill in the art could not predict which [polymerase] could be mutated to be appropriately temperature sensitive **and** non-cytopathic, or what mutations would be required for this." Applicants respectfully submit that a person of ordinary skill in the art could make and use additional nucleic acids encoding mutant polymerases without undue experimentation.

The claims as amended specify that the nucleic acid encodes a polymerase of alphaviral origin. As discussed above, alphaviruses (e.g., Sindbis virus, Aura virus, Venezuelan equine encephalitis virus, and SFV) share a high degree of homology. Indeed, mutation of the same Glycine residue in SFV and Sindbis virus (residues Gly153 and Gly154, respectively, of nsp4) renders the polymerase temperature sensitive. Additional temperature-sensitive mutations in the SFV RNA dependent RNA polymerase were known as of the filing date of the present application (see, e.g., Hahn *et al.*, *J. Virol.* 63:1194-1202 (1989)). Likewise, additional mutations producing non-cytopathic SFV and Sindbis replicons were identified using methods known as of the filing date (see, e.g., Perri *et al.*, *J. Virol.* 74:9802-9807 (2000) and Frolov *et al.*, *Proc. Natl. Acad. Sci. USA* 93:11371-11377 (1996)).

Contrary to the implications of the Office Action, non-cytopathic mutations in nsp2 can be combined with temperature-sensitive mutations in nsp4 to produce a temperature-sensitive, non-cytopathic replicon. Indeed, Lundstrom *et al.* successfully combined non-cytopathic mutations in nsp2 with temperature sensitive mutations in nsp4, indicating that the combination of non-cytopathic mutations in nsp2 and temperature-sensitive mutations in nsp4 is additive (see Lundstrom *et al.*, *Histochem.*

Cell Biol. 115:83-91 (2001)). Thus, the Lundstrom *et al.* reference shows that the method taught in the present specification can be used with SFV to yield a temperature-sensitive, non-cytopathic replicon. Accordingly, a person of ordinary skill in the art could make and use a number of non-cytopathic, temperature-sensitive SFV replicons in accordance with the invention.

The Office Action states that, "[w]hile it is simple to construct nucleic acids which would comprise both types of mutations, the characteristics of these novel polypeptides would be highly unpredictable." To the contrary, a person of ordinary skill in the art would reasonably expect that mutations leading to non-cytopathic and temperature-sensitive phenotypes would have an additive effect. Viral non-structural proteins are often produced as polyproteins, such as nsp 1-4 of Sindbis virus or SFV, or nsp 1-5 of Kunjin Flavivirus. These polyproteins then are further processed by proteolysis. For Sindbis virus, expression of nsp1-3 and nsp 3-4 as two separate polyproteins results in RNA replication and transcription (Lemm *et al.*, *J. Virol.* 67:1905-1915 (1993); copy enclosed), indicating that nsp2 and nsp4 need not be located on the same polypeptide chain for proper protein folding. Thus, the combination of mutations occurring either far apart on the primary sequence, or on different polypeptide chains have a high probability of having an additive effect. Indeed, as shown by the Lundstrom reference, non-cytopathic mutations in nsp2 can be additively combined with temperature-sensitive mutations in nsp4. Thus, by combining (i) non-cytopathic mutations, which can easily be made and screened for as described in the specification (at page 21, lines 23-20) or in the prior art (e.g., Frolov or Agapov references) with (ii) temperature-sensitive mutations, which also can readily be made and screened for as

described in the specification (at page 22, lines 21-25), a person of ordinary skill in the art can produce nucleic acids encoding non-cytopathic, temperature-sensitive RNA polymerases without undue experimentation.

The Office Action states that Schnizer *et al.*:

teach an example in which mutations of two separate amino acids of the yeast F1-ATPase beta subunit were combined and produce totally unpredictable results. Specifically, one mutation at position 203 and four different mutations at position 211 were found to inactivate and destabilize the F1-ATPase complex when expressed separately. However, when the position 203 mutation was combined with and [sic] any one of the position 211 mutations in the same construct, destabilization was suppressed and activity was restored to the ATPase complex.

For several reasons, Applicants respectfully submit that the Schnizer reference is not dispositive of the effects of mutations on the claimed nucleic acids. In contrast to the implications of the Schnizer reference, the present application and the Lundstrom reference clearly demonstrate the additive effects of the mutations in the claimed nucleic acids.

The additive effect of mutations has also been described for the L protein of respiratory syncytial virus, in which mutations that are approximately 500 amino acids apart contributed additively to temperature sensitivity and to attenuation (see, Juhasz *et al.*, *Vaccine* 17:1416-1424 (1999)). Similarly, mutations scattered throughout a protein have also been shown to have an additive effect on protein stability (see, Serrano *et al.*, *J. Mol. Biol.* 233:305-312 (1993)). This is in contrast to the mutations described in the Office Action for the Yeast F1-ATPase, described by Schnizer, in which two mutations were in close proximity in the primary structure, and - more important - close in the tertiary structure. That the structural effect of one mutation can be compensated by

mutating another side chain in close proximity is well known (see, Blaber *et al.*, *J. Mol. Biol.* 246: 317-330 (1995)).

Applicants also note that Schnizer *et al.* suggest that the L203F mutation in yeast-F1 ATPase is destabilizing based on the authors' failure to isolate the mutant ATPase using one particular method that is effective for isolating the wild-type enzyme. The Schnizer *et al.* reference, however, fails to consider the possibility that the authors' failure to isolate the enzyme may not be due to protein stability, but rather to alternative requirements for protein purification. Furthermore, the activity of the L203F mutant was not tested in the chloroform extraction assay where the other mutants at position 211 showed activity, which declined over time. In fact, it is conceivable that the L203F mutation is a stabilizer of the active site of the F-ATPase as suggested by the reduction of *K_m* below the wild-type value (see Table II), in which case the combinations of mutations at positions 211 and 203 would be at least partially additive.

In sum, the specification and the prior art provide sufficient guidance to allow a person of ordinary skill in the art to produce nucleic acids encoding non-cytopathic, temperature-sensitive RNA dependent RNA polymerases of alphaviral origin without undue experimentation. Additionally, a person of ordinary skill in the art could construct vectors comprising promoters that are recognized by such polymerases. A person of ordinary skill in the art of molecular biology would know to combine a vector comprising a promoter with a polymerase that recognized that promoter. Although some experimentation may be necessary to make and use the invention, the amount of experimentation is not *undue*. Just as a person of ordinary skill in the art is prepared to screen hybridoma clones to obtain a monoclonal antibody, a person of ordinary skill in

the art is prepared to screen various combinations of mutations to obtain the claimed nucleic acids encoding non-cytopathic, temperature sensitive polymerases, and that person would be prepared to screen various combinations of promoters and polymerases. As with screening for hybridomas, there is a very high probability of obtaining the desired polymerases, as shown by the examples described above. Accordingly, the enablement requirement has been satisfied and the rejection for lack of enablement should be withdrawn.

Claims 75-101 and 103-125 were rejected for alleged indefiniteness. This rejection has been obviated by the foregoing amendment, which indicates that the promoter is "recognized" by the polymerase. Withdrawal of this rejection is respectfully requested.

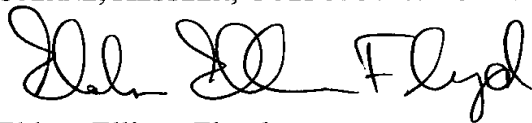
Conclusion

All of the stated grounds of objection and rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding objections and rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

STERNE, KESSLER, GOLDSTEIN & FOX P.L.L.C.

A handwritten signature in black ink, appearing to read "Eldora Ellison Floyd". The signature is fluid and cursive, with the first name "Eldora" being more prominent.

Eldora Ellison Floyd
Attorney for Applicants
Registration No. 39,967

Date: July 31, 2001

1100 New York Avenue, N.W.
Suite 600
Washington, D.C. 20005-3934
(202) 371-2600

Version with markings to show changes made

Claims 80, 85, 104, and 108 have been canceled.

75. (Amended) A DNA molecule which encodes an RNA molecule comprising:

- (a) at least one *cis*-acting sequence element,
- (b) a first open reading frame which encodes a non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase of alphaviral origin, and
- (c) at least one second nucleotide sequence selected from the group consisting of:
 - (i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;
 - (ii) a sequence complementary to all or part of the second open reading frame of (i); and
 - (iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is [activated] recognized by said non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase.

81. (Amended) The DNA molecule of claim [80] 75, wherein the RNA-dependent RNA polymerase is derived from a Sindbis virus.

103. (Amended) A DNA vector system comprising one or more polynucleotides which encode RNA molecules, said RNA molecules comprising:

- (a) at least one *cis*-acting sequence element,
- (b) a first open reading frame having a nucleotide sequence encoding a non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase of alphaviral origin, and
- (c) at least one second nucleotide sequence selected from the group consisting of:
 - (i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;
 - (ii) a sequence complementary to all or part of the second open reading frame of (i); and
 - (iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is [activated] recognized by said non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase.

125. (Amended) A composition comprising one or more RNA molecules, said RNA molecules comprising:

- (a) at least one *cis*-acting sequence element,
- (b) a first open reading frame having a nucleotide sequence encoding a non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase of alphaviral origin, and
- (c) at least one second nucleotide sequence selected from the group consisting of:
 - (i) a second open reading frame encoding a protein, or portion thereof, wherein said second open reading frame is in a translatable format after one or more RNA-dependent RNA replication events;
 - (ii) a sequence complementary to all or part of the second open reading frame of (i); and
 - (iii) a sequence encoding an untranslated RNA molecule, or complement thereof;

wherein said second nucleotide sequence is operably linked to a promoter which is [activated] recognized by said non-cytopathic, temperature-sensitive RNA-dependent RNA polymerase.

New claims 126-136 have been added.



RECEIVED
AUG 02 2001
TECH CENTER 1600/2900

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

Renner *et al.*

Appl. No.: 09/275,883

Filed: March 25, 1999

For: Inducible Alphaviral Gene
Expression System

Art Unit: 1632

Examiner: R. Schnizer

Atty Docket: 1700.0020001/JAG/EEF

Declaration Under 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Marco Boorsma, do hereby declare and say:

1. I am a graduate of the University of Gronigen (The Netherlands), where I obtained the degree of Doktorandus (Drs.) in June 1998, and I was previously a research scientist at Cytos Biotechnology, the assignee of the above-captioned application.

2. The experiments described herein provide evidence that the pCYTts vector functions in a non-cytopathic, temperature sensitive manner in a variety of mammalian cell lines. Certain experiments described below employed the vector eCYTts, which is a derivative of pCYTts that contains a puromycin resistance marker under the control of a SV40 promoter. The vector eCYTts was non-cytopathic and temperature-sensitive in a variety of mammalian cell types. These assays were carried out as follows.

3. The cell lines BHK21 C13-2P, BHK21, CHO-K1, HEK 293, and HEK 293T were cotransfected using LIPOFECTAMINE 2000 (LF 2000) with (i) the vector eCYTts containing the coding sequence for secreted alkaline phosphatase (eCYTts-SEAP (Fig. 1)) and (ii) the marker construct pEGFP, encoding enhanced green fluorescent protein. At one day prior to transfection, cells were plated in their normal growth medium containing serum and without antibiotics; they were 90-95% confluent on the day of transfection. Suspension cells were prepared at a concentration of 1.5×10^6 cells per 6-wells (2 ml). If the normal growth medium was a medium other than DMEM, DMEM containing 10% fetal calf serum was substituted for the normal growth medium at two hours prior to transfection. For each well of cells to be transfected, 3.6 μ g eCYTts-SEAP and 0.4 μ g pEGFP were diluted into 250 μ l OptiMEM I supplemented with glutamax.

4. For each well of cells, the appropriate amount of LF 2000 reagent was diluted into OptiMEM I supplemented with glutamax and incubated for 5 minutes at room temperature. For CHO-K1 cells, 12.5 μ l of LF2000 were used; 10 μ l of LF2000 were used for HEK 293 cells; and 15 μ l of LF2000 cells were used for BHK21 cells. The diluted DNA was combined with the diluted LF2000 reagent by gentle mixing, and then incubated for 20 minutes at room temperature. The resulting solution then was added to each well of cells, covering the whole well, and the cells were incubated at 37°C. At 5 hours after transfection was initiated, the cell medium was replaced with 2 ml of normal growth medium, and the cells were further incubated at 37°C.

5. One day after transfection, the cells were split at the following ratios 1:4, 1:10, and 1:20, then incubated at 37°C. On the second day after transfection, the cells that were split

1:4 and 1:10 were shifted to 29°C in order to induce SEAP expression from the temperature-sensitive vector. The cells that were split 1:20 remained at 37°C for comparison.

6. At 5 days after transfection, the SEAP activity of the conditioned medium of each well was determined as follows. The conditioned media was heated to 65°C for 5 minutes. The media then was centrifuged at 14,000 rpm for 30 seconds, and 50 µl of each supernatant was transferred into a 96-well plate. A 50 µl aliquot of FAST™ p-Nitrophenylphosphate was added, and absorbance was measured at 405 nm every 30 seconds for 20 minutes at 37°C using a Benchmark microplate reader. After plotting the data, the slope of the linear part of the curve was determined, giving the SEAP activity in 50 µl of conditioned medium. The SEAP activity of the entire well was determined by multiplying the activity in 50 µl by 40.

7. The cells then were harvested and split into two aliquots. In one aliquot, viable cells were counted using a CEDEX cell counter. The second aliquot was used for determining the percentage of transfected cells using a FACS Calibur. The SEAP activity per transfected and per viable cell was calculated.

8. The obtained data indicate that the vector eCYTts-SEAP, a derivative of pCYTts, is non-cytopathic and induces temperature-sensitive gene expression. Table 1, below, shows that SEAP activity was detected in the supernatants of cell cultures that had been induced at 29°C to express SEAP. SEAP expression was measured in BHK21, BHK21-C13-2P, HEK 293, HEK293T, and CHO-K1 cells at 7 days post-transfection. While SEAP activity was detected in supernatants of cells that were temperature-shifted to 29°C, SEAP activity was not detected

supernatants of the same cotransfected cells that were maintained at 37°C, indicating that the vector functioned in a non-cytopathic, temperature-sensitive manner.

Table 1: Cells transiently cotransfected with eCytTS SEAP and pEGFP

Cell Line	SEAP Activity of Supernatant After 64 hrs at 29°C [mOD/(min*1000,000 cells)]	Viable Cells (100,000 cells)	Viability (%)
BHK21 C13-2P	0.49	5.54	88.5
BHK21	0.28	39.64	94.9
HEK293t	0.16	25.37	95.4
HEK 293	0.20	8.18	92.2
CHO-K1	0.13	4.62	94.7

9. In similar experiments, the vectors eCYTts and pCYTts were used to obtain inducible expression of GFP in various mammalian cells. The vector eCYTts-GFP was used to express GFP in BF cells in a non-cytopathic, temperature-sensitive manner. As shown in Fig. 2, transfected cells that were induced at 29°C for 48 hours expressed GFP, as detected by fluorescence microscopy. Likewise, the vector eCYTts-AID56-GFP was used to express an AID56-GFP fusion protein in C2C12 myoblasts in a non-cytopathic, temperature-sensitive manner. AID56-GFP is a fusion protein of the amyloid- β precursor protein intracellular protein and green fluorescent protein. As shown in Fig. 3, expression from the eCYTts vector was induced at 29°C for 48 hours, and expression of the AID-GFP fusion protein was detected by fluorescence microscopy. As shown in Fig. 4, GFP expression was also induced and detected in CHO-K1 cells that were stably transfected with CYTts-GFP and induced at 29°C for 10 days.

Additionally, inducible GFP expression was detected in COS-7 cells that had been stably transfected with pCYTts-GFP (data not shown).


10. Further evidence that the vector pCYTts is non-cytopathic is provided by the enclosed Fig. 5. In this assay, HEK 293 cells were transfected with the vector pMut1-GFP-IRESpuro (Fig. 5A). This vector is similar to pCYTts, but lacks the ts6 mutation that renders the polymerase activity temperature sensitive. Cells transfected with pMut1-GFP-IRESpuro were grown under puromycin selection for 2 weeks (Fig. 5B), and essentially all of the cells in the clone produced GFP, as detected by fluorescence microscopy (Fig. 5C). Thus, this experiment shows that the pCYTts vector is non-cytopathic, contrary to the findings of Agapov *et al.*, *Proc. Natl. Acad. Sci., USA* 95:12989-12994 (1998).

11. Still further evidence that the pCYTts vector can be used in a non-cytopathic, temperature-sensitive manner is provided by the enclosed publication by Boorsma *et al.*, *Nature Biotechnology* 18:429-432 (2000). The pCYTts vector was used to obtain inducible expression of genes encoding SEAP, GFP, β -interferon, and erythropoietin in CHO-K1 cells, COS-7 cells, C2C12 myoblasts, and BHK cells as well as to obtain inducible expression of genes encoding RIP death domain in BHK cells.

12. In sum, the data described herein support the assertion that the vector pCYTts can be used to obtain inducible expression of a gene of interest in a non-cytopathic, temperature-sensitive manner.

13. THAT, I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both under § 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of this document or any patent associated herewith.

Date 22/07/01


Dr. Marco Boorsma